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## Review

## Regulating cellular oxygen sensing by hydroxylation

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Abstract

Oxygen homeostasis under conditions of limited O<sub>2</sub> supply requires hypoxia-dependent gene regulation. The transcription factor complex hypoxia-inducible factor 1 (HIF-1) has been recognized as the master regulator that mediates the adaptational genetic response to ensure restoration of energy supply. This review will focus on the recent advances in understanding the hypoxia-induced cellular response with particular respect to cellular O<sub>2</sub> sensing for adequate control of HIF-1 activation.

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**Keywords:** HIF-1; Hypoxia; Hypoxia-induced gene expression

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1. O<sub>2</sub> sensing via HIF $\alpha$  factors

Crucial to linking reduced oxygen supply to changes in gene expression are hypoxia-inducible transcription factors, or HIFs [1]. Across the animal kingdom, HIF is a heterodimer of  $\alpha$ - and  $\beta$  subunits. Both subunits belong to the family of basic-helix-loop-helix (bHLH)/PAS<sup>1</sup> transcription factors. Under physiological oxygen partial pressures ( $pO_2$ ), HIF is quickly destabilized. In contrast,  $\alpha$  and  $\beta$  subunits heterodimerize in the nucleus in response to oxygen deprivation. Here, the dimer specifically binds to target gene motifs called hypoxia response elements (HREs) to either induce or suppress gene expression. A decade after the seminal identification of HIF-1 as the responsible factor for the hypoxia-induced transcription of the red cell producing hormone erythropoietin [2], this transcription factor has now been implicated to possibly control even up to several hundreds (i.e. ~2–5% of genome) of hypoxia-responsive

genes in humans [3]. In mammals, the 70 HIF targets validated to date [4] typically fall into two main categories whose functions aim to restore energy and O<sub>2</sub> homeostasis by either cell-autonomous or systemic means:

- (a) increasing anaerobic energy production via stimulated glycolytic substrate flux;
- (b) improving tissue oxygenation via stimulated angiogenesis, vasodilation and erythropoiesis.

HIF signaling – although not the only pathway linking declining  $pO_2$  with changes in DNA activity (see Cummins and Taylor [5] for a recent review on HIF-independent transcription responses to hypoxia) – has thus been appreciated as a major component in the homeostatic adaptations and survival of cells under both physiological and tumorigenic-pathological oxygen limitations [6]. Notwithstanding this key importance for hypoxic survival, the mechanisms and players behind this switch from active (hypoxia) to inactive (normoxia, reoxygenation) states of HIF signaling were only poorly understood until recently. Fortunately, there has been a remarkable progress made during the last 5 years, advancing our comprehension of

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<sup>1</sup> PAS: acronym for PER, ARNT, SIM, the first proteins discovered to contain this domain.

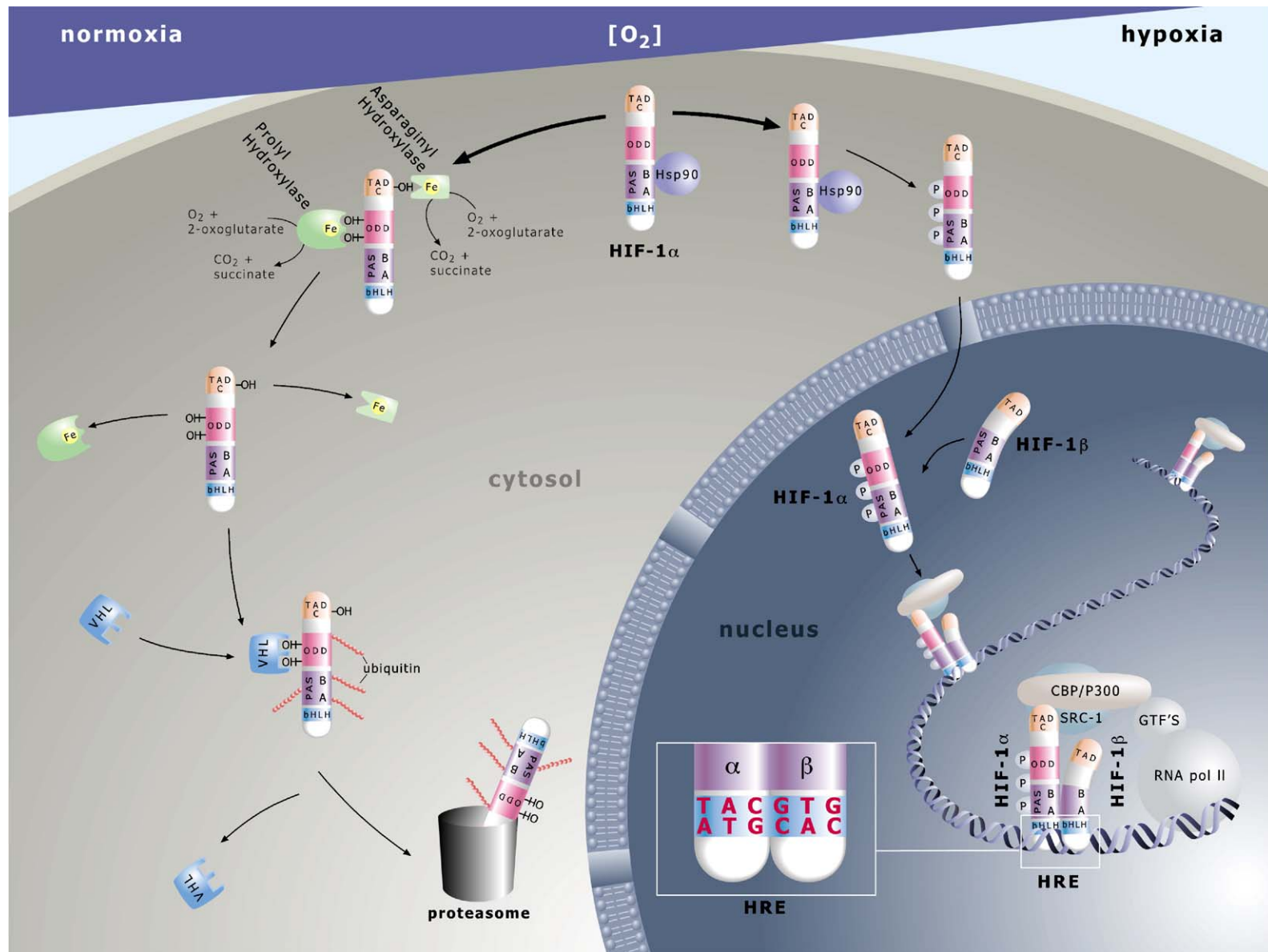


Fig. 1. HIF- $\alpha$  mediated pathway of O<sub>2</sub> sensing. Molecules are not drawn to scale. Prolyl hydroxylase in the figure represents PHD1–3. For further details see text.

cellular oxygen sensing onto an entirely new level. To record this recent progress, with a particular focus on the hydroxylase-mediated regulation of the HIF signaling pathway, is the goal of this current review.

From nematodes to crustaceans to flies to vertebrates, HIF activity is controlled at the protein level through oxidative modifications of the  $\alpha$  subunits [7]. Multiple isoforms of these regulatory HIF proteins exist in fish, amphibians, birds and mammals [8]. Three HIF- $\alpha$  subunits (HIF-1 $\alpha$ , -2 $\alpha$  and -3 $\alpha$ ) have been reported in human and rodent cells, with five and six splice variant transcripts known to occur for the human 1 $\alpha$  and 3 $\alpha$  isoforms, respectively [9,10]. All three  $\alpha$  subunit factors share the ability to heterodimerize with the ubiquitously expressed HIF-1 $\beta$  subunit, originally known as the aryl hydrocarbon receptor nuclear translocator or ARNT, thus producing HIF-1, -2 and -3. While both stability and activity of the 1 $\alpha$ , 2 $\alpha$  and some 3 $\alpha$  (i.e. 3 $\alpha_1$ –3 $\alpha_3$ ) subunits are quickly impaired by normoxia or reoxygenation, the steady state levels of the ARNT protein are unaffected by changes in oxygen tension [11]. As outlined in detail below, and summarized in Fig. 1, the HIF oxygen sensors, that control the abundance of HIF- $\alpha$  proteins in the cell are a family of novel prolyl hydroxylases named PHD1–3, for the characteristic prolyl hydroxylase domains contained within the reading frame [12–14]. In the presence of oxygen, these PHDs catalyze the Fe(II)-dependent hydroxylation of specific prolyl residues (Pro<sup>402</sup> and Pro<sup>564</sup> in human HIF-1 $\alpha$ ) contained within the oxygen-dependent degradation domain (ODD) of HIF-1 $\alpha$  [15], -2 $\alpha$  or -3 $\alpha_{1-3}$  subunits (see Fig. 1: ODD hydroxy-prolines as “OH”). Once hydroxylated, the HIF- $\alpha$  subunits rapidly bind to the von Hippel-Lindau (pVHL) tumor suppressor protein that is the recognition component of an E3 ubiquitin ligase complex, thereby tying prolyl hydroxylation to ubiquitination and proteasomal degradation of these regulatory constituents under high or rising  $pO_2$  (e.g. [16,17]; see Fig. 1). In addition, acetylation of ODD-contained lysines (e.g. K<sup>532</sup> in human HIF-1 $\alpha$ ) via acetyltransferases such as ARD1 *might* be another, though currently far more controversially discussed, enzymatic trigger for enhanced pVHL recognition and destabilization of alpha subunits [18–20]. Unlike PHDs, the activity of acetyltransferases is not known to be influenced by changes in oxygen tension, thus making it hard to see how normoxia should lead to differentially high rates of protein substrate acetylations (for review see [21,22]). Notwithstanding our incomplete mechanistic understanding, the efficacy of these hydroxylation- (and potentially acetylation-) based post-translational controls can best be appreciated by the <5 min half-life of HIF-1 $\alpha$  upon reoxygenation [11] and, conversely, the instantaneous accumulation of the transcription factor in vitro [23] and in vivo [24] during declining oxygen tensions.

A second O<sub>2</sub>-requiring hydroxylation, that of a single asparagine (Asn<sup>803</sup> in human HIF-1 $\alpha$ ) within the C-terminal transactivation domain of HIF-1 $\alpha$  and -2 $\alpha$  (see Fig. 1: TAD-C, hydroxyl-asparagine as “OH”) by an asparaginyl hydroxylase dubbed FIH-1 (factor inhibiting HIF-1), leads

to steric hindrance of the interaction between  $\alpha$  subunits and the coactivator proteins p300/CBP (note: lack of TAD-C domains in 3 $\alpha$  isoforms). This hindrance prohibits the transactivation of target genes under high  $pO_2$  [25,26]. The absolute requirement of PHD- and FIH-1 activities for oxygen and Fe<sup>2+</sup> [27–29] has made these hydroxylation reactions susceptible to inhibition not only by declining  $pO_2$  but also by hypoxia-mimicking agents such as transition metals (e.g., Co<sup>2+</sup>) and iron chelators (e.g., desferrioxamine, DFO; ciclopiroxolamine (CPX) [12,30]). Long before the discovery of PHD-mediated O<sub>2</sub> sensing, Co<sup>2+</sup>, DFO and CPX were empirically known to be able to induce erythropoietin, i.e. HIF target, gene responses under normoxia [31,32]. As a strength of concept, insights into the catalytic requirements of PHDs can now incorporate these earlier data into a plausible model of HIF control. Upon exposure to hypoxia, cobalt or iron chelators, hydroxylation of prolyl and asparaginyl residues is reduced (see Fig. 1). This inhibition enables the  $\alpha$  subunit to escape proteolytic degradation and, assisted by various interacting factors (e.g. heat shock protein 90, HSP 90) [4,33] and/or stimulatory kinase signals (reviewed in [6]), to efficiently translocate into the nucleus where the phosphorylated form (P) dimerizes with ARNT (reviewed in [22]) through intermolecular interactions between the HLH and PAS domains (see Fig. 1). Next, the  $\alpha$ : $\beta$  heterodimer associates with the transcriptional coactivators p300/CBP and Src-1 to regulate target gene transcription via HRE binding sites. Specific recognition of HRE motifs is accomplished through the N-terminal basic (b) region in both factors (see Fig. 1: 5' TACGTG 3' given as HRE example; note:  $\alpha$  subunit occupancy of 5' TAC nucleotides, and  $\beta$  subunit binding to 3' GTG nucleotides). The HIF/coactivator complex is now endowed to modulate the activity of the general transcription factor (GTF)/RNA polymerase II machinery in a hypoxia-responsive fashion.

## 2. Regulation of HIF $\alpha$ by hydroxylation

As mentioned above, both the regulation of O<sub>2</sub>-dependent lability and thus protein abundance as well as transcriptional activity of HIF- $\alpha$  subunits depend on hydroxylation. So far, PHD1, PHD2, PHD3 and FIH-1 have been identified to perform this post-translational modification. They now appear as today's best candidates for cellular HIF oxygen sensors. A fourth prolyl hydroxylase termed PH-4 has been isolated from a database approach [34]. Although this protein showed great similarity to the known PHDs and was able to suppress HIF-accumulation and HIF-dependent reporter gene activation, it failed to show activity in an HIF-1 $\alpha$ -pVHL in vitro interaction assay. Therefore the role as an HIF prolyl hydroxylase, particularly with O<sub>2</sub> dependent activity, is currently not clear.

Initially, the search for HIF-associated O<sub>2</sub> sensors focused on heme proteins that would – in response to altered states in oxygenation – change their heme conformation and valency and thereby generate a signal to control O<sub>2</sub>-dependent gene



expression [1,31]. However, the post-translational hydroxylation of HIF- $\alpha$  implied O<sub>2</sub>-dependent enzymatic activity. Subsequently, it was demonstrated that modification of proline residues in the ODD of HIF- $\alpha$  controls abundance while asparagine hydroxylation is the important switch for regulating the binding of coactivator p300/CBP [17,35]. Conservation of the O<sub>2</sub>-sensing system down to *C. elegans* helped to define a new family of dioxygenases that regulate HIF- $\alpha$  by prolyl hydroxylation [12,13,36]. Although the human homologues were variously named HPH-1/EGLN3/PHD3, HPH-2/EGLN1/PHD2, and HPH-3/EGLN2/PHD1 [37] we will use the initial term PHD1, -2, and -3. Shortly thereafter the enzyme hydroxylating the TAD-C previously termed FIH-1 was identified as an asparaginyl hydroxylase that fell into the same family of dioxygenases [26].

By definition, dioxygenases metabolize molecular oxygen and incorporate both atoms of oxygen into their products. PHDs and FIH-1 are Fe<sup>2+</sup>, 2-oxoglutarate (2OG) and ascorbate dependent non-heme binding oxygenases of which the enzymatic activity is regulated by O<sub>2</sub> availability (see Fig. 1). With respect to cellular localization and O<sub>2</sub> regulated enzymatic activity they differ from the well-known collagen prolyl hydroxylase (C-P4H1) whose  $\alpha$ (I) subunit is a HIF-1 target gene [38]. Like PHDs and FIH-1, the C-P4H1 releases CO<sub>2</sub> and succinate upon completion of the enzymatic cycle and its activity depends on substrate and ascorbate concentration as it is well known from scurvy. However, the most significant difference related to their function was found when the affinity for O<sub>2</sub> was determined for the PHDs and FIH-1 and compared to C-4PH1. While the K<sub>m</sub> value for O<sub>2</sub> of the C-P4H1 was around 40  $\mu$ M, values initially reported for the PHDs were between 230 and 250  $\mu$ M which exceeds even the concentration of O<sub>2</sub> in air [39]. For FIH-1, the K<sub>m</sub> was found to be considerably lower, 90  $\mu$ M [40], but still more than twice as high as that of C-P4H1 (40  $\mu$ M; [41]). These K<sub>m</sub> values were determined using the <sup>14</sup>CO<sub>2</sub> release from labeled 2OG catalyzed by recombinant enzymes and short 19 amino acid (aa) HIF- $\alpha$  peptides as substrates. A caveat with respect to these K<sub>m</sub> measurements has been expressed since interpretation of the O<sub>2</sub> affinity would need to consider the reaction kinetics of the enzymes [42]. In general, information on the kinetics of prolyl hydroxylases is mainly based on data from C4-PH1, but not from PHDs. In C4-PH1, O<sub>2</sub> appears to be bound first to the enzymatic complex. Subsequently, the enzyme–O<sub>2</sub> complex is stabilized by binding pro-collagen polypeptide substrate [43]. More recently, this order of binding was questioned. Instead, it was proposed that a preformed enzyme–substrate complex binds O<sub>2</sub> [44], which would be in line with crystallography data on FIH-1, where such a preformed enzyme–Fe<sup>2+</sup>–2OG–substrate complex was described to bind O<sub>2</sub> [45]. This order of binding implies, however, that substrate bound to the enzyme could affect the K<sub>m</sub> for O<sub>2</sub>. Indeed, when the length of HIF- $\alpha$  peptides used for in vitro assays was extended to 200 aa the K<sub>m</sub> value for O<sub>2</sub> of the PHDs dropped to 100  $\mu$ M (Johanna Myllyharju,

personal communication, 2006). Whether full length HIF- $\alpha$  proteins would lower the K<sub>m</sub> even further is still a matter of debate. Full length HIF- $\alpha$  proteins are almost insoluble and have not been used in the in vitro enzymatic measurements.

Even a K<sub>m</sub> of 100  $\mu$ M corresponds with the maximal O<sub>2</sub> concentrations in the body, the alveolar *p*O<sub>2</sub> in the lung. Oxygen tensions in the tissue, particularly in the kidney where HIF signaling is required for erythropoietin expression [46], or in the placenta where HIF is required for regular development [47], are certainly lower than any of the K<sub>m</sub> values determined so far. Measurement of tissue *p*O<sub>2</sub> under physiological conditions has been notoriously difficult. Most of the technical *p*O<sub>2</sub> sensors, e.g. of Clark-type, consume oxygen and thus blur measurements of *p*O<sub>2</sub>. In addition, tissue *p*O<sub>2</sub> is determined by the ratio of the rate of consumption to the rate of supply which may vary even within tissue areas very close to each other [48]. However, a range of oxygen concentration from 40 down to 4  $\mu$ M may reasonably be assumed. As such, the high K<sub>m</sub> for oxygen would place the steep slope range of the activity curve determined for PHDs and FIH-1 right into the range of physiological *p*O<sub>2</sub> values. Any incremental reduction in the *p*O<sub>2</sub> would thus trigger a marked lowering of hydroxylation capacity [42]. This was demonstrated already in the first description of PHDs when a decrease in HIF hydroxylation between *p*O<sub>2</sub> values of 150 mm Hg and 75 mm Hg was found [12]. Interestingly, the K<sub>m</sub> of FIH-1 is lower than for PHDs and indeed, experiments indicate that FIH-1 control dominates HIF activation during exposures to a lower *p*O<sub>2</sub> range [49]. The emerging picture of graded hydroxylase activities that operate with maximal sensitivity in the range of physiological tissue *p*O<sub>2</sub> thus fits initial in vitro observations, where half-maximal activation of HIF signaling was reported at oxygen concentrations of 15 to 20  $\mu$ M [50]. Detection of nuclear HIF-1 $\alpha$  in cells, under conditions where the pericellular *p*O<sub>2</sub> was measured and oxygen consumption was taken into account [51], indicates that HIF accumulation may be achieved at higher *p*O<sub>2</sub> values but that, in addition, cell-specific differences may exist [52]. Collectively, however, there is no doubt that the absolute requirement for O<sub>2</sub> causes lack of enzymatic activity under anoxia. Upon a graded decrease of the *p*O<sub>2</sub> within the physiological range overall hydroxylation capacity of PHDs and FIH-1 will become limiting and thus the overall process of hydroxylation will be rate-limiting in the system that regulates HIF abundance and activity. As such, the enzymes fulfill the criteria of oxygen sensors. In addition, the *p*O<sub>2</sub> range in which the oxygen sensors work may be affected by abundance of the enzymes themselves [42]. This would, in fact, explain different oxygen sensitivity for cells in culture under identical physical conditions [52].

### 3. Hydroxylase abundance and interacting proteins

Prolyl- and asparaginyl-hydroxylases are non-equilibrium enzymes because they only catalyze the hydroxylation of

their substrate but not the reverse reaction. Thus the abundance of HIF-hydroxylases will determine HIF- $\alpha$  accumulation and activity at distinct  $pO_2$ 's. Expression of the PHD iso-enzymes varies between tissues but no specific role for this difference has been determined in vivo [53]. PHD1 is highly expressed in the testis whereas PHD3 mRNA is abundant in the heart. A recent study using newly developed monoclonal antibodies determined protein distribution throughout several organs and revealed tissue specific distribution and also differences in staining intensity between normal and malignant tissue [54]. Knock-out mice for PHD1 to 3 have been generated and are currently characterized with respect to their phenotype. Homozygous PHD2 $^{-/-}$  mice are not viable, but PHD1 $^{-/-}$  and PHD3 $^{-/-}$  are. An interesting phenotype was reported for PHD1 $^{-/-}$  mice which showed a significant protection from hypoxia induced muscle damage in a hind limb ischemia model (P. Carmeliet, Keystone Symposium January 2006, Breckenridge, CO).

A strong stimulus that regulates PHD2 and PHD3 abundance is hypoxia itself. Initially it was observed that the half-life of HIF-1 $\alpha$  was significantly shortened after prolonged or severe hypoxic periods [55]. It was found that the pVHL/HIF pathway regulated hypoxic inducibility of PHD2 and 3 [56] and recently functional HREs were isolated and characterized in the promoter of the PHD2 gene [57] as well as of the PHD3 gene [58]. In contrast, PHD1 is not induced by hypoxia, potentially even reduced in a particular subset of cells [59]. Several negative regulatory elements in the PHD1 promoter, and a possible role for ARNT in this hypoxic down-regulation, have been reported [60]. Likewise, levels of FIH-1 are not affected by hypoxia [61]. Hypoxic induction of PHD2 and 3 therefore forms a negative feedback loop whose function is not yet entirely clear. It has recently been suggested that higher levels of hydroxylase expression may extend the regulatory range of the oxygen sensors to more severe hypoxia [42]. On the other hand, induction, notably of PHD3, appears to be of particular importance for the reoxygenation phase when higher PHD3 levels may increase the dynamics of the hypoxic response by shortening the half-life of HIF-1 $\alpha$  protein. Very recently, two alternatively processed PHD3 transcripts, designated PHD3 $\Delta 1$  and PHD3 $\Delta 4$  were identified [62]. The expression of both PHD3 and PHD3 $\Delta 1$  was observed in all tissues and cell lines tested, although the expression of the novel PHD3 $\Delta 4$  appeared to be restricted to primary cancer tissues. The function of PHD3 $\Delta 4$  was assessed in transfection experiments showing a preserved prolyl hydroxylase activity although the impact on  $O_2$  sensing remains to be fully elucidated.

Another open question is whether the localization of PHDs is affected after hypoxic induction. PHD enzymes show a distinct intracellular distribution pattern with PHD1 mostly, if not exclusively, localized in the nucleus whereas PHD2 predominantly resides in the cytoplasm and PHD3 is found in both cellular compartments [61]. A recent study

using novel antibodies more or less confirmed the localization of PHD2 and 3 while PHD1 was also found in the cytoplasm [54]. This may indicate that cell-specific differences exist or that particular circumstances affect the translocation of the oxygen sensors. It has for example been described that PHD1 is estrogen-dependent although no effects on subcellular localization were studied so far [63]. Very recent work, however, provides evidence that cellular localization is a dynamic process, particularly that PHD2 may also be found in the nucleus upon induction [64]. Irrespective of the cellular localization and the tissue distribution, the current consensus is that PHD2 is the main enzyme that regulates steady state levels of HIF-1 $\alpha$ . This is indicated by the fact that knock-down of PHD2 by siRNA is sufficient to increase HIF-1 $\alpha$  levels under normoxic condition [65]. In addition, HIF target genes were up-regulated indicating that inhibition of PHD2 alone is sufficient for HIF-1 target gene activation although FIH-1 activity was not reduced. This notion has further been tested in a study where the relative importance of PHD2 at different levels of hypoxia was found to be most important for normoxic and moderately hypoxic situations whereas FIH-1 appears to regulate HIF activity at more severe hypoxia [49]. This fits in fact the lower  $K_m$  value for oxygen of FIH-1 as mentioned above.

Most probably, however, regulation of PHDs comprises several more levels of complexity. PHD activity is inhibited when intracellular calcium is chelated although no obvious calcium binding site has been identified [66]. This could indicate that other calcium dependent cofactors are required for full PHD activity. The ring finger E3-ubiquitin ligases Siah1a/2 were recognized to co-immunoprecipitate with PHD1 and 3 [67]. Siah1a/2 appear to particularly affect PHD3 levels by increased proteolysis. Siah1a/2-deficient cells showed higher levels of PHD3 and a loss of hypoxic HIF-1 $\alpha$  induction which could be reversed by siRNA against PHD3. Interestingly, Siah proteins themselves are hypoxia-inducible suggesting that, under hypoxic conditions, PHD3 proteolysis will increase. On the other hand, PHD3 is HIF-1 $\alpha$  target gene and PHD3 expression is induced in hypoxia [58,61]. Thus hypoxia increases expression and degradation of PHD3 which in turn controls HIF-1 $\alpha$  levels. How this intricate regulation and counter-regulation affects physiological oxygen sensing is unclear at present although Siah2 $^{-/-}$  mice subjected to hypoxia displayed an impaired erythropoietic response which might indicate physiological relevance of this regulation [67].

When the initial biochemical purification of an HIF-1 prolyl hydroxylase revealed a 320–440 kDa complex that appeared much larger in mass than individual PHDs [68], it became obvious that other proteins associate with PHDs. In an attempt to characterize such proteins, the chaperonin complex TriC (aka CCT) was found to co-purify with PHD3. Whether PHD3 represents a folding substrate for TriC is currently not clear [69]. Interaction of PHD2 with OS-9, a widely expressed protein of yet unassigned function, appears

to increase PHD2 activity and thus to promote degradation of HIF-1 $\alpha$  [70]. Knock-down of OS-9 by siRNA increased HIF-1 $\alpha$  protein levels, HIF-1-mediated transcription, and expression of the HIF-1 target gene VEGF under non-hypoxic conditions. Interaction of the tumor suppressor protein ING4 with PHD2 surprisingly did not affect either PHD2 activity or HIF stability [71]. Instead, ING4 binding to PHD2 appears to interfere with transcriptional activation of HIF-1 $\alpha$  either by interacting with the TAD-N domain within the ODD or through hindrance of the correct folding of the active HIF complex between the C-terminus and the DNA binding domains [71]. This field is now under active investigation, particularly with respect to other PHD targets [72]. In this regard, the groups of R. Wenger and D. Katschinski recently reported several yeast-two-hybrid based identifications of novel PHD1 (i.e. onconeural cerebellar degeneration-related protein 2, Cdr2), PHD2 (i.e. peptidyl-prolyl cis/trans isomerase FK506-binding protein 38, FKBP38) and PHD3 (i.e. activating transcription factor-4, ATF-4) interacting factors that might either represent PHD hydroxylation targets (Cdr2, ATF-4) or PHD activity modulators (FKBP38) (see *Acta Physiol.* 2006, vol. 186, suppl. 1, PM07P-4, PM08A-5, PM08A-7). Moreover, the novel WD-repeat protein Morg1 was identified to act as a molecular scaffold for PHD3 [73]. Morg1 was shown to support the specific activity of PHD3 and may even be important for maximal activity of the enzyme under certain conditions.

#### 4. Effect of divalent metal ions on PHD activity

Classically it had been reported that cobalt chloride can mimic the anemic or hypoxic induction of erythropoietin production and in fact cobalt chloride was used as an erythropoietic agent [74]. Later, in vivo and in vitro studies revealed that this effect is due to cobalt chloride dependent induction of HIF-1 [46]. Since nickel (Ni<sup>2+</sup>) and manganese (Mn<sup>2+</sup>) acted in a similar way and induced erythropoietin production it was initially proposed that those divalent transition metal ions would replace iron in the potential heme protein sensor, thereby locking this protein in its active deoxy conformation [31]. This concept has been perpetuated when the Fe<sup>2+</sup>-dependent PHD enzymes were identified as O<sub>2</sub> sensors and is currently used to explain why divalent metal ions increase HIF-1 $\alpha$  protein, even though PHDs turned out to be non-heme binding dioxygenases (i.e. iron is directly coordinated with the protein). It has indeed been found that hypoxia mimetics like Co<sup>2+</sup> and iron chelators inhibit PHD activity [12]. However, a competing hypothesis has been proposed, whereby cobalt and nickel deplete intracellular ascorbate, which is also required for full enzymatic activity (see below; [75]). Nevertheless, the absolute requirement of Fe<sup>2+</sup> for HIF hydroxylase activity is beyond doubt. When recombinant PHD enzymes were recently purified and tested for their K<sub>m</sub> values it was found that iron was so tightly bound to these enzymes that K<sub>m</sub>

values for iron could only be estimated with lower than 0.1  $\mu$ M [76]. For FIH-1, a K<sub>m</sub> for iron of 0.5  $\mu$ M was measured, which still is considerably lower than the K<sub>m</sub> of 2  $\mu$ M for C-P4H1. Although the values for PHDs were only estimated, high affinity binding of the metal by PHDs is supported by the finding that, in vitro, the iron chelator desferrioxamine (DFO) and the divalent metal ions (Co<sup>2+</sup>, Ni<sup>2+</sup>, Mn<sup>2+</sup>) were more effective in inhibiting FIH-1 than PHD preparations (see Fig. 1). However, the obvious and important question whether cellular iron availability affects physiological hydroxylase function has not yet been fully addressed. One recent report found changes in iron distribution in response to NO treatment and may thus indicate that cellular iron distribution is in fact able to affect oxygen sensing [77]. Although the direct addition of iron to cultures reduced HIF-1 $\alpha$  levels, it may be doubted whether this iron is in fact transferred to the PHD without prior oxidation and may thus affect other yet unknown ways of HIF- $\alpha$  accumulation [78].

#### 5. Substrate specificity, ascorbate requirement and ROS

Both in HIF-1 $\alpha$  and HIF-2 $\alpha$  the proline residues to be hydroxylated are in the context of an LXXLAP consensus sequence. This sequence is not recognized by C-P4H1, but by PHDs. Within this consensus are the two critical proline residues 402 and 564 in human HIF-1 $\alpha$  which are both recognized by PHD1 and 2 [79], but not by PHD3. PHD3 will only hydroxylate proline 564. In addition, Leu574 in human HIF-1 $\alpha$  was found to be essential for pVHL binding [80] and PHD2 recruitment to the Pro564 hydroxylation site [81]. High substrate specificity of PHDs to recognize these particular proline residues, therefore, results from the conserved presence of the LXXLAP consensus together with adjacent 'recruiter residues'. Consequently, the K<sub>m</sub> for 19 aa model peptides including the consensus sequence was as low as 7 to 8  $\mu$ M [39]. Despite this high substrate specificity, in vitro mutagenesis studies unexpectedly revealed that prolyl hydroxylation is rather tolerant towards a range of substitutions in the vicinity of the target proline [79]. It has been reasoned that these effects may be due to high concentration of short peptides used for in vitro studies. Therefore X-ray structure studies of PHDs and HIF- $\alpha$  protein substrate are mostly needed. This was so far only achieved for FIH-1 bound to the TAD-C of HIF [45]. Here the higher stringency and particularly the requirement for valine 802 for hydroxylation activity became obvious when an extensive set of hydrogen bonds of the TAD-C was found to be formed with FIH-1 to bring the asparagine 803 close to the catalytic iron of the active site of the enzyme [45]. Moreover, structural changes have been observed between a state where the TAD-C is bound to FIH-1, which would cause hydroxylation and inhibition of activation, and an active state where p300 is bound to the TAD-C [82,83]. Collectively, recognition of the substrate, both by PHDs and FIH-1, appears to depend on multiple residues within the



surrounding protein which ensure highly specific substrate recognition.

Ascorbate has been proposed to protect prolyl hydroxylases from uncoupled reactions where no substrate is hydroxylated and reactive oxygen species (ROS) may be generated that could damage the enzymes [41]. In fact, ascorbate deficiency has been found to be limiting for PHD function in human tumor cells causing high HIF-1 $\alpha$  levels [78]. Usually C-4PH1 enzymes catalyze the uncoupled decarboxylation of 2OG at a rate of less than 1% of the complete reaction [41]. When proline is hydroxylated, a number of cycles can be run without ascorbate, but then Fe<sup>3+</sup> needs to be reduced for continued activity of the enzyme. In the case of uncoupled reactions, e.g. if no available substrate is recognized, ascorbate may act as an alternative O<sub>2</sub> acceptor to limit the generation of reactive oxygen species. Under these conditions, ascorbate is consumed stoichiometrically. For other 2OG-dependent oxygenases the generation of ROS has been described and can inactivate the related oxygenases by self-oxidation which might cause fragmentation of the enzymes [84]. So far, uncoupling has not been observed for PHDs, but about 1% of the maximum activity of FIH-1 did not account for hydroxylation in the presence of an HIF substrate [40]. While a great percentage of uncoupled reactions may be harmful to the enzymes and potentially other targets in the cell as pointed out above, the precise mechanism is not fully elucidated. One particular open question is whether the HIF $\alpha$  substrate binds prior to or after oxygen has been bound to the enzyme. On the one hand, only substrate-bound protein would bind oxygen and thus substrate recognition would indeed reduce the potential for ROS generation. On the other side, oxygen affinity may well be modulated by substrate binding as pointed out above [37]. Another under-investigated issue concerns our limited understanding when comparing the regulation of HIF-hydroxylase activities between organisms capable of *de novo* ascorbate synthesis (e.g. rodents) and others for which vitamin C is an essential nutrient (e.g. humans, primates, guinea pigs).

With respect to ROS it is noteworthy that the model of cellular oxygen sensing by oxygen-dependent enzymatic activity of PHDs is in competition with the hypothesis that mitochondria and/or other cellular oxidases generate ROS to control oxygen dependent gene expression [85]. Reduced levels of ROS, particularly H<sub>2</sub>O<sub>2</sub>, have been observed under hypoxia and were correlated with increased HIF-dependent erythropoietin expression [86]. This was later associated with a perinuclear Fenton type reaction that reduced HIF-1 $\alpha$  levels and would thus indicate that locally increased ROS diminish HIF activation [87]. On the other hand, ROS have been found to inhibit PHD activity which would explain how ROS can increase HIF-1 $\alpha$  levels [88]. Increased ROS production by mitochondria, however, has often been achieved by the use of inhibitors of mitochondrial respiration which have recently been shown to completely alter cellular oxygen supply [89]. Due to diffusion limited oxygen availability to cells in culture [51], any inhibition of oxygen

consumption would significantly alter pericellular oxygen tensions. As such, it has recently been proposed that inhibition of the mitochondrial electron transport chain would not necessarily produce reactive species that act as signaling molecules, but could instead increase oxygen availability for PHD enzymes [89]. So far, it has not been possible to clearly define intracellular O<sub>2</sub> levels and, particularly, O<sub>2</sub> gradients that occur with lowest values around mitochondria where oxygen is consumed. However, an interesting scenario has been proposed for the action of nitric oxide (NO) with respect to inhibition of HIF accumulation under hypoxic conditions. NO is known to inhibit mitochondrial O<sub>2</sub> consumption. Under the influence of NO, oxygen, which is no longer consumed by the mitochondria, may be redistributed to PHD enzymes causing increased activity and thus HIF-1 $\alpha$  degradation [90]. Similar mechanisms may account for inhibition of HIF- $\alpha$  accumulation under the influence of inhibitors of mitochondrial respiration or depletion of mitochondrial DNA ( $\rho$ 0 cells). This would not require the action of ROS for oxygen sensing and will explain results that were recently reported to substantiate this hypothesis [91,92]. Although a direct effect of ROS on Fe<sup>2+</sup> in PHD enzymes was claimed [88], this finding contradicts the observation that upon reoxygenation where, without doubt, major amounts of ROS are produced, rapid degradation of HIF- $\alpha$  due to PHD activity is not impaired.

## 6. Linking oxygen sensing with tricarboxylic acid cycle activity

Recently some exciting reports on the effects of tricarboxylic acid cycle (TCA) intermediates affecting PHD activity have been published [93–95]. Since PHDs are 2OG-dependent enzymes and since 2OG is an intermediate in the TCA, an obvious follow-up question is: Does cross-talk between these two important cellular systems affect oxygen sensing capacity? While the K<sub>m</sub> values for 2OG of PHDs are in the range of 55–60  $\mu$ M, and for FIH-1 around 25  $\mu$ M [39,40], the exact 2OG concentration within the cell or even in a subcellular compartment is difficult to calculate. It was therefore important to dissect that hereditary cancer syndromes associated with defects in succinate dehydrogenase (subunits B, C or D) displayed competitive inhibition of PHD activity through increased levels of succinate [93]. While this would biochemically be referred to as product inhibition, another hereditary cancer syndrome where fumarate hydratase is deficient, revealed that increased fumarate levels are likewise able to significantly inhibit 2OG-dependent PHD activity and thus increase HIF-1 $\alpha$  levels [94]. In addition, in an earlier study where the role of co-substrates for prolyl hydroxylases was investigated, it was found that the endogenous 2-oxoacids pyruvate and oxaloacetate can both act as competitive inhibitors of 2OG-dependent oxygenases [96]. More recently, pyruvate and oxaloacetate have also been found to induce HIF-1 [97]. In



summary, the effect of intermediates of the citric acid cycle (TCA intermediates, see Fig. 1) on oxygen sensing provides an explanation for the pathology observed with defects in these hereditary cancer syndromes. Whether this cross-talk is also found under physiological circumstances will be an interesting field of investigation.

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